REMARKS

The August 22, 2003 Official Action has been carefully considered. In view of the amendment submitted hereinwith and these remarks, favorable reconsideration and allowance of this Application are respectfully requested.

At the outset, it is noted that among the pending claims, claims 1-6, 10, 11, 13, 17, 19, 20, 22-25, 27-31, and 33-39, claims 2, 6, 10, 11, 13, 17, 19, 20, 22-25, and 29-31 are withdrawn from consideration; claims 27, 28, 33, 35, and 36 are allowed; and claims 1, 3-5, 34, and 37-39 are rejected.

It is also noted that the Examiner has indicated that Applicants fail to comply with USPTO rules for receiving the benefit of an earlier filing date under 35 U.S.C. 119(e). In a telephonic interview with the Examiner conducted on August 27, 2003, the Examiner acknowledged the receipt of the specification amendment that perfected the priority claims. Accordingly, this objection is now moot.

At page 3 of the present Official Action, the Examiner rejects claims 34 and 37 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement.

Additionally, at page 4, the Examiner rejects claims 1, 3-5, 38, and 39 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement.

The foregoing constitutes the entirety of the rejections raised by the Examiner in the August 22, 2003 Official Action.

In accordance to the present claim amendment, claim 37 has been amended to recite that the carrier is "selected from the group consisting of controlled release film, nanoparticle, and microparticle." Support for this amendment can be found at page 16, lines 12-13 of the specification. No new matter is introduced by the present claim amendment, entry of which is respectfully requested.

In light of the foregoing amendment and the following remarks, each of the above-noted rejections under 35 U.S.C. §112, first paragraph is respectfully traversed.

Claim 34 And Claim 37, As Presently Amended, Fully Comply With The Written Description Requirement Of U.S.C. §112, First Paragraph

The Examiner states that the originally filed specification fails to support the recitation of "vascular smooth muscle cell" in claim 34, and the recitation of "a carrier that permits controlled release of" tenascin C in claim 37.

First, the Examiner's attention is directed to Examples 1, 2, and 3 at pages 27-29 of the present specification, wherein rat arterial smooth muscle cells, i.e. A10 cells, were used to demonstrate the ability of denatured collagen, Tenascin C, or cytochalasin D in enhancing the efficiency of delivery of nucleic acids into these vascular smooth muscle cells. Accordingly, the recitation of "vascular smooth muscle cell" in claim 34 is fully described in the present specification.

Moreover, by the claim amendments presented herewith, claim 37 has been amended to recite that the carrier is "selected from the group consisting of controlled release film, nanoparticle, and microparticle." Support for this amendment can be found at page 16, lines 12-13 of the specification.

Inasmuch as the subject matter of claim 34s and presently amended claim 37 is fully described in the present specification, the 35 U.S.C. §112, first paragraph rejection of these claims is improper and should be withdrawn.

Claims 1, 3-5, 38, And 39 Fully Comply With The Enablement Requirement Of U.S.C. §112, First Paragraph

At pages 4-5, the Examiner rejects claims 1 and 3-5, 38, and 39 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement. Particularly, it is the Examiner's position that the specification, while being enabling for enhancing transfection of cultured cells with cationic liposomes comprising plasmid by growth in the presence of tenascin C, fails to provide enablement for any other embodiments embraced by these claims. This rejection is respectfully traversed for the following reasons.

From page 16, line 23, through page 18, line 17 of the specification, an enabled description of various nucleic acid delivery systems for transfecting nucleic acids into cells is provided. Specifically, the Examiner's attention is directed to the paragraph begins at page 16, line 26, ends at page 17, line 5 of the specification, wherein it is sated:

> "The nucleic acid delivery system can be any of the nucleic acid delivery systems described herein, or others known or to be known in the art. Various nucleic acid delivery systems are described, for example in Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; and Ausubel et al., 1994, Current Protocols in Molecular Biology, John Wiley & Sons, New York. Such nucleic acid delivery systems comprise the desired nucleic acid, by way of example and not by limitation, in either "naked" form as a "naked" nucleic acid, or formulated in a vehicle suitable for delivery, such as in a complex with a cationic molecule or a liposome forming lipid, or as a component of a vector, or a component of a pharmaceutical composition."

Additionally, at page 9, lines 18-26, the specification provides:

> "[A] "nucleic acid delivery system" means any composition or system, known or to be known in the art, which can be used to deliver a nucleic acid

> into a cell. ... By way of example, and not by limitation, such systems include the following: "naked" (i.e. non-complexed) nucleic acids; nucleic acids complexed with cationic molecules such as polysine and liposome-forming lipids; vectors such as a plasmid vector, a viral vector, an antisense oligonucleotide, an adenovirus, an adeno-associated virus, a retro-virus, a lentivirus, a herpesvirus, and a bacteriophage; and a linearized nucleic acid."

Therefore, the specification, as it was originally filed, has fully described and enabled the transfection of nucleic acids, either "naked" or formulated in any vehicle delivery system, into cells.

Moreover, the specification also provides ways of delivering the nucleic acids and/or the agent that enhances transfection into cells both *in vitro* and *in vivo*. Particularly, as it is specified at page 18, lines 11-17 of the specification:

> "Preferably, the agent and/or nucleic acid delivery system are provided (e.g. administered) in a manner which enables tissue-specific uptake of the agent and/or nucleic acid delivery system. Preferred techniques include using tissue or organ localizing devices, such as wound dressings or transdermal delivery systems, using invasive devices such as vascular or urinary catheters, and using interventional devices such as stents having delivery capability and configured as expansive devices or stent grafts."

Thus, the specification is fully enabling for the enhancement of transfection of cells, both *in vitro* and *in vivo*, with the compositions of the present invention.

Further, as it is stated in International Standard Elec. Corp. v. Ooms, 157 F.2d 73,70 USPQ 32 (D.C. Cir. 1946):

> "The specification need describe the invention only in such detail as to enable a person skilled in the most relevant art to make and use it. When an invention, in its different aspects, involves distinct arts, that specification is adequate which enables the adepts of each art, those who have the

> best chance of being enabled, to carry out the aspect proper to their specialty."

In the instant case, methodologies of delivering nucleic acids and/or the agent that enhances transfection into cells both *in vitro* and *in vivo* are widely understood and used by those skilled in the art. This is evidenced by Perlstein et al., in "DNA Delivery from an Intravascular Stent with a Denatured Collagen-Polyactic-Polyglycolic Acid-controlled Release Coating: Mechanisms of Enhanced Transfection", *Gene Therapy* 2003;10:1420-1428, a copy of which is enclosed herewith. Notably, several of the present inventors are co-authors of this reference. In Perlstein, the authors utilized denatured-collagen-PLGA-coated stents (either with GFP-plasmid loading or control without vector) into pig coronary arteries and demonstrated the ability of denatured collagen to enhance cell transfection *in vivo*. (See page 1423, right column, under the section "Denatured collagen enhances DNA delivery from coronary stents *in vivo*" and page 1427, right column, under section "Transfection via stent: *in vivo*".)

Even further, as stated in Fiers v. Sugano, 984 F.2d 1164,25 USPQ 2d 1601,1607 (Fed. Cir. 1993):

> "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of §112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. …" "[A]ny party making the assertion that a U.S. patent specification or claims fails, for one reason or another, to comply with §112 bears the burden of persuasion in showing said lack of compliance."

In the instant case, the specification has provided detailed teachings of delivering nucleic acids, either "naked"

or in compositions, into cells, either *in vitro* or *in vivo*. The presence of the agents enhancing cell transfection. The Examiner has failed to form a *prima facie* case of unenablement. The rejection of claims 1, 3-5, 38, and 39 is therefore improper and should be withdrawn.

Conclusion

It is respectfully requested that the amendments presented herewith be entered in this application, since the amendments are primarily formal, rather than substantive in nature. This amendment is believed to clearly place the pending claims in condition for allowance. In any event, the claims as presently amended are believed to eliminate certain issues and better define other issues which would be raised on appeal, should an appeal be necessary in this case

In view of the amendments and remarks presented herewith, it is respectfully urged that the rejections set forth in the August 22, 2003 Official Action be withdrawn and that this application be passed to issue. In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any issues outstanding may be resolved through a telephone interview, the Examiner is requested to telephone the undersigned attorney at the phone number given above.

Respectfully submitted,

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SKILLMAN, P.C.
Agent for Applicants

By _____
Tong Li
Registration No. 47,748

Enclosure:

- Perlstein et al., *Gene Therapy* 2003;10:1420-1428

Gene Therapy (2003) 10, 1420–1428

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DNA delivery from an intravascular stent with a denatured collagen-polylactic-polyglycolic acid-controlled release coating: mechanisms of enhanced transfection

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We previously demonstrated that DNA–polylactic–polyglycolic acid (PLGA)-coated stents can deliver genes to the arterial wall with reporter expression involving 1% of neointimal cells. The present study investigated a novel formulation utilizing denatured collagen in DNA-stent coatings; denatured collagen was hypothesized to enhance gene transfer due to adhesion molecule interactions and actin-related mechanisms. Arterial smooth muscle cells (SMCs) cultivated on denatured collagen had significantly greater plasmid DNA (β-galactosidase) transfection than SMC grown on native collagen (18.3± 1.2 vs 1.0± 0.1%, P< 0.001). The denatured-collagen effect was completely blocked with anti-$\alpha_v\beta_3$ integrin antibody. SMCs cultivated on native collagen supplemented with tenascin-C (TN-C), a protein recognized by $\alpha_v\beta_3$ integrins, showed a 33-fold increase in transfection compared to control (P< 0.001); this effect was also blocked with anti-$\alpha_v\beta_3$ antibody. We observed that cells grown on denatured collagen had marked F-actin-enriched stress fibers and intense perinuclear G actin, compared to those grown on native collagen, which demonstrated F-actin-enriched focal adhesions without perinuclear G-actin localization. Cytochalasin-D, an F actin depolymerizing agent, caused significantly increased SMC transfection in cells cultivated on native collagen compared to control cells (18.0± 1.8 vs 3.02± 0.9%, P< 0.001) further supporting the view that actin-related cytoskeletal changes influence transfection. A denatured-collagen–PLGA composite vascular stent coating similarly resulted in increased plasmid DNA green fluorescent protein (GFP) expression compared to controls (P< 0.001) in SMC cultures; the increased transfection was blocked by anti-$\alpha_v\beta_3$ antibody. Pig coronary studies comparing denatured-collagen–PLGA-coated stents containing plasmid DNA (encoding GFP) to coated stents without DNA demonstrated 10.8% of neointimal cells transfected; this level of expression was almost an order of magnitude greater than previously reported with a DNA delivery stent. It is concluded that denatured collagen incorporated into plasmid DNA-stent coating formulations may increase the level of gene expression in vitro and in vivo because of integrin-related mechanisms and associated changes in the arterial smooth muscle cell actin cytoskeleton.
Gene Therapy (2003) **10,** 1420–1428. doi:10.1038/sj.gt.3302043

Keywords: *site specific; $\alpha_v\beta_3$ integrins; actin cytoskeleton*

Introduction

Plasmid DNA delivery from intravascular stents[1] is a forefront approach for achieving gene therapy for cardiovascular disease. Controlled release of DNA from vascular stents was previously investigated by our group in a series of studies using green fluorescent protein (GFP) plasmid DNA incorporated into a polylactic–polyglycolic acid copolymer (PLGA) emulsion coating on stainless-steel stents.[1] These studies demonstrated GFP expression in cultured vascular SMCs, as well as in pig coronary arteries, with 1% of neointimal arterial smooth muscle cells transfected. Although these results demonstrated great promise, the relatively low level of transfection achieved in this initial study would likely preclude therapeutic efficacy. Therefore, the present paper focused on a mechanistic approach toward enhancing the level of transfection with DNA-delivery stents.

Prior investigations have demonstrated that specific cell adhesion receptors, including integrin heterodimers, can be used to target uptake of either plasmid DNA[2,3] or viral[4,5] vectors. However, these studies did not examine whether the addition of exogenous ligands to the extracellular matrix (ECM) affected gene expression

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efficiency. Previous studies[6] have demonstrated that denatured type I collagen, but not native collagen, can upregulate Tenascin C (TN-C) in arterial smooth muscle cells (SMCs). TN-C is an ECM protein that enhances cell survival and proliferation.[7,8] Furthermore, this denatured collagen–SMC interaction can be blocked with an anti-β3 integrin antibody,[6,9] thereby indicating a specific integrin receptor interaction is responsible for this effect. In this study, we attempted to determine whether specific ECM–integrin interactions involving denatured-collagen coatings and associated actin-cytoskeletal changes would increase the extent of plasmid DNA transfection. We hypothesized that denatured-collagen coatings on a DNA-delivery stent could lead to an increased level of arterial gene expression from DNA-delivery stents.

The present study investigated DNA-controlled release using denatured collagen as a component of a DNA-delivery stent coating to hypothetically increase the level of plasmid DNA transfection through mechanisms involving β_3 integrin receptor interactions and associated changes in actin dynamics. The goals of our investigations were:

(1) To investigate the effects of a denatured-collagen substrate (an $\alpha_v\beta_3$ integrin ligand) on plasmid DNA transfection in cell culture, compared to a native type I collagen substrate
(2) To determine if the observed transfection was related to β_3 integrin interactions;
(3) To learn if TN-C, an alternative $\alpha_v\beta_3$ integrin ligand, could also positively influence transfection efficiency in cell culture
(4) To examine changes in the F and G-actin cytoskeletal distribution due to denatured collagen in culture, and to determine whether actin-specific agents affect transfection
(5) To formulate and characterize a coating that includes denatured collagen for use in a DNA-controlled release stent to deliver plasmid DNA to pig coronary arteries

Results

SMC cultivated on denatured vs native collagen demonstrate higher levels of transfection

When A10 cells were cultured on denatured collagen (Figure 1b), they demonstrated a 17-fold greater β-galactosidase transfection level then did A10 cells grown on native collagen (Figure 1a), 18.3 ± 1.2 vs $1.01\pm0.1\%$ respectively, $P<0.001$ (Figure 1e). Cell viability approached 100% (99.7 ± 0.1 vs $99.5\pm0.1\%$ respectively) on both substrates, as measured by trypan blue exclusion, and confirmed by live/dead assay of cells at 72 h (Figures 1c and d). Although there was no difference in cell adherence (12.2 ± 0.9 vs 12.3 ± 0.8 cells/mm^2 respectively at 5 h), cells cultivated on denatured collagen had a greater proliferation rate than did cells grown on native collagen, with 3.1 ± 0.7-fold more cells after 72 h compared to A10 cells cultivated on native collagen per WST-1 assays. This metabolic assay was corroborated by direct visual count of cells per mm^2 on each substrate (40.2 ± 3.2 vs 16.0 ± 0.6 cells/mm^2, respectively, in a parallel experiment). Nevertheless, the increase in transfection efficiency far exceeded the enhanced effects on growth (17.0- vs 3.1-fold).

Since denatured collagen is a known $\alpha_v\beta_3$ integrin ligand,[6,10,11] we next investigated the involvement of this receptor in modulating denatured-collagen-mediated transfection. Using integrin-function-blocking antibodies, we found that pretreating the cells with anti-$\alpha_v\beta_3$ monoclonal antibodies had no significant effect on transfection of cells cultured on native collagen ($P=0.78$, data not shown), but eliminated the enhanced transfection observed when A10 SMCs were maintained on a substrate of denatured collagen (from $18.3\pm1.2\%$ to $2.9\pm0.2\%$ $P=0.001$). Cell growth was not reduced by addition of the antibody (22.0 ± 0.1 vs 23.9 ± 1.8 cells/mm^2 in a representative 72 h experiment). Nonimmune mouse IgG had no effect on reducing the transfection efficiency ($17.9\pm0.4\%$, P=n.s.; Figure 1e), or on cell growth.

Next, we determined whether the $\alpha_v\beta_3$ integrin-dependent effects on enhanced transfection were specific for SMC interactions with denatured collagen, or whether alternate $\alpha_v\beta_3$ integrin ligands (ie TN-C) would also affect the efficiency of transfection. Addition of TN-C to a native collagen substrate resulted in a dose-dependent increase in transfection (Figure 2a). However, while the lowest dose of TNC-C used, 1-μg/ml, had no effect on transfection efficiency (Figure 2a), it resulted in a three-fold increase in proliferation per WST-1 assay (data not shown). This suggests that although TN-C also caused increased SMC proliferation in these studies, enhanced transfection and proliferation are independent events. The highest dose of TN-C used, 50-μg/ml, caused a 33-fold increase in transfection efficiency with only an associated seven-fold increase in proliferation (data not shown). Furthermore, like denatured-collagen-mediated transfection, the TN-C-mediated transfection enhancement was found to be $\alpha_v\beta_3$ dependent. Blocking antibody studies using either anti-$\alpha_v\beta_3$ or anti-β_3 or reduced transfection to levels seen without the addition of TN-C (Figure 2b).

Actin changes associated with increased transfection in vitro

Since changes in cell interactions with ECM via integrins ultimately control intracellular actin dynamics, we next investigated the relation between cellular DNA uptake and the cytoskeletal distribution of the different forms of actin. Double fluorescent label studies were performed using phalloidin (rhodamine) as a marker of F actin, and nuclease-I (FITC) as a marker of G-actin. Cells grown on native collagen were somewhat elongated and displayed prominent colocalization of F and G actins in focal adhesions in confocal images (Figure 3a). In A10 cell cultures grown on denatured collagen, we also observed a marked qualitative increase in G-actin intensity and concentration around the nucleus (Figure 3b), and prominent F-actin stress fibers, not noted in native collagen cultures.

Having shown an association between cell substrate, actin depolymerization (G actin), and transfection capacity, we sought to perform experiments to investigate a potential causal relation between manipulation of the actin cytoskeleton and transfection capacity. The F-actin

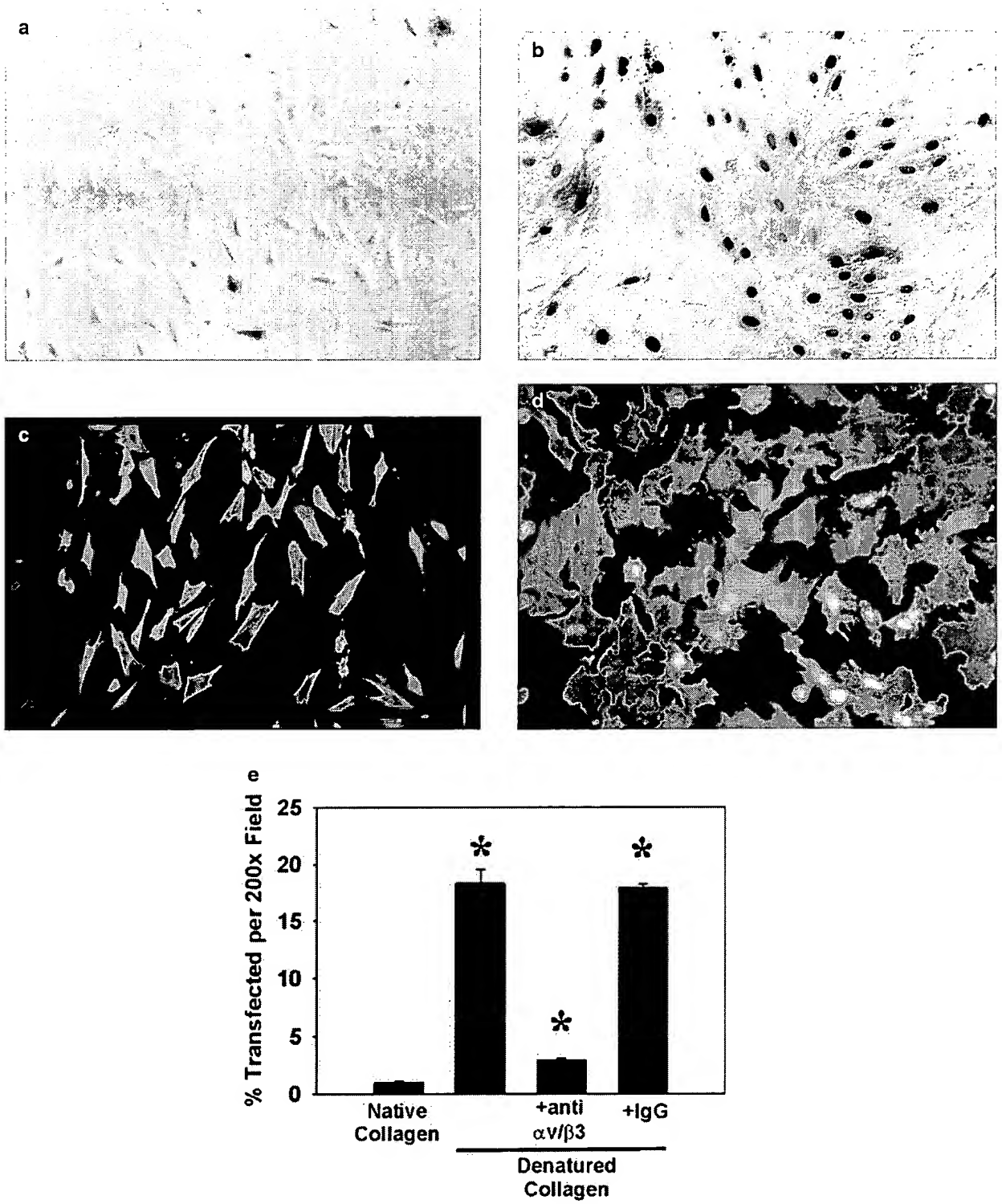

Figure 1 *Cell morphology and transfection on native or denatured collagen. (a) A10 cells grown on native collagen assume an elongated morphology and poor transfection efficiency, as indicated by the paucity of β-gal-expressing cells (dark blue). (b) Cultures grown on denatured collagen are denser, and show high transfection efficiency, indicated by the many β-gal-expressing cells. (c) A10 cells grown on native collagen (live/dead staining) with high viability per enzymatic metabolism of calcein (green fluorescence), vs retention of EthD-1 by dead cells (red fluorescence), similar to (d) A10 cells grown on denatured collagen. (e) Quantitation of cells expressing β-gal confirms significant enhancement with culture on denatured collagen, while specific antibody blockade of $\alpha_v\beta_3$ integrin cell–substrate interaction on denatured collagen reduces the transfection rate to that of cells on native collagen. Nonspecific IgG (control) had no effect on transfection. (a, b) bright-field photomicrographs of X-gal-stained cultures, (c, d) fluorescence photomicrographs of live/dead assay™ stained cultures, × 200 magnification. *P<0.001.*

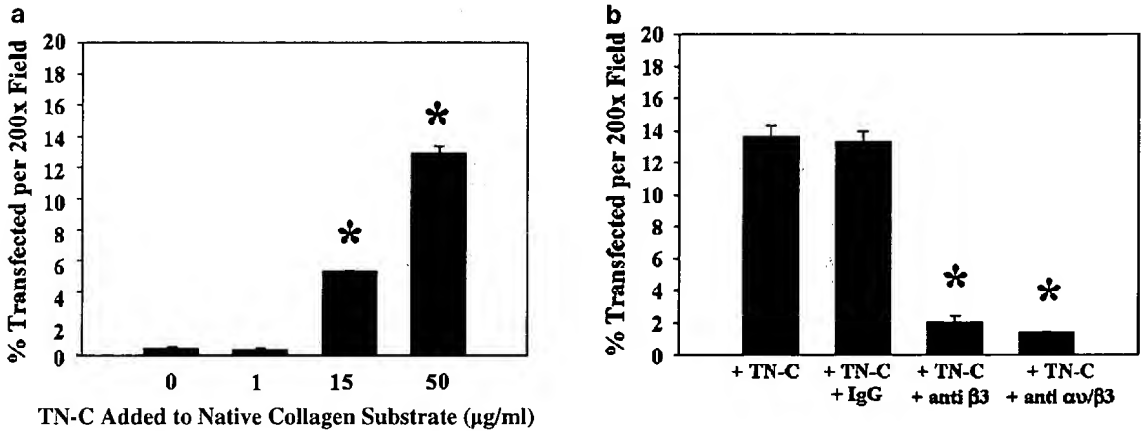

*Figure 2 Quantitation of TN-C-enhanced transfection and its dependence on $\alpha_v\beta_3$ integrin cell-native collagen interaction. (a) The addition of TN-C to a native collagen substrate causes a dose-dependent increase in transfection at concentrations at or above 15 μg/ml (*P < 0.001). (b) Use of either anti-α_v/β_3 or anti-β3 antibody significantly reduces the high transfection rate seen in the presence of TN-C in native collagen (*P < 0.001); nonspecific IgG did not differ from control.*

destabilizer cytochalasin-D, at a dose of 100 nM (Figure 4a), significantly enhanced transfection efficiency when compared to control cells maintained on native collagen (18.0±1.8% vs 3.02±0.9%, Figure 4b) without significantly affecting cell proliferation (30.1±3.9 vs 23.8±5.7 cells/mm², means±s.e., at 72 h, respectively). Moreover, the cytochalasin-D effect could be blocked via the pretreatment of the A10 cell cultures with 10 μM jasplakonilide (Figure 4b), an F-actin stabilization agent, known to counteract the F-actin depolymerization effects of cytochalasin-D (1.14±0.54%, $P<0.001$, Figure 4b). Jasplakonilide did not reduce cell proliferation (30.9±4.2 cells/mm²).

Denatured-collagen stent-coating formulation

Having demonstrated in cultured SMCs that denatured collagen enhances the transfection efficiency of plasmid DNA, we next carried out experiments in which plasmid DNA and denatured collagen were formulated as a coating on vascular stents. It should be noted that native collagen gels, because of their fragile nature, proved to be unsuitable for stent coatings; these preparations broke up upon stent deployment with immediate shedding of the native collagen coating. Thus, no native collagen control group was possible for the *in vivo* studies.

In the present study, crown stents (Cordis, Morristown, NJ, USA) were coated with several formulations, comparing DNA dispersed in denatured collagen, to the same coating, but with an outer PLGA polymer-coating layer. These studies demonstrated (Figure 5a) that the release rate of plasmid DNA from the denatured-collagen-only coating was far too rapid for potential *in vivo* use, with virtually all of the DNA released in less than 30 min. However, successively increasing concentrations of an outer PLGA coating resulted in a more sustained release of DNA, with a release duration of more than 24 h (Figure 5a). In addition, it was necessary to crosslink the denatured-collagen coatings in order to retain these coatings as a stable robust surface layer on the vascular stents. Thus, all denatured-collagen-coated stents were treated with water-soluble carbodiimide. The plasmid DNA released from these formulations in sustained elution studies was intact, as demonstrated by agarose gels (Figure 5b).

A series of cell culture studies was carried out with vascular stents coated with the DNA-denatured collagen, plus PLGA. We observed transfection localized only to cells in culture in contact with the stent struts (data not shown), and thus confined our observations to those areas. In these experiments, we also sought to learn if the $\alpha_v\beta_3$ integrin was involved, especially since the coating formulation involved crosslinking denatured collagen followed by coating with PLGA, and thus differed markedly from the pure-denatured type I collagen cell culture substrates studied in the earlier experiments. Nevertheless, A10 cell culture blocking antibody studies using monoclonal anti-$\alpha_v\beta_3$ demonstrated significant inhibition of the $\alpha_v\beta_3$ enhanced transfection associated with the denatured-collagen–PLGA coating (Figure 5c). However, the relative magnitude of the transfection *in vitro* with denatured-collagen–PLGA stents, and its inhibition, was less than that observed with A10 cells plated directly on the collagen substrates, indicating the likely influence of other formulation steps involving the PLGA and crosslinking procedures. Nevertheless, this particular formulation demonstrated enhanced transfection in cell culture compared to controls, and furthermore, unlike native collagen, was suitable as a stent coating for intravascular studies with DNA-delivery stents.

Denatured collagen enhances DNA delivery from coronary stents in vivo

GFP expression levels in the arterial wall with denatured-collagen–PLGA-coated stents exhibited high levels of GFP fluorescence expression in the neointima (Figures 6a–c vs control artery 6d), which was confirmed by GFP immunohistochemistry (Figure 6e vs control artery 6f). Denatured-collagen–PLGA-coated stents in the present studies containing 500 μg DNA demonstrated 10.4±1.23% neointimal cells expressing GFP. Coated stents without plasmid DNA (controls) demonstrated no evidence of GFP expression (Figure 6d).

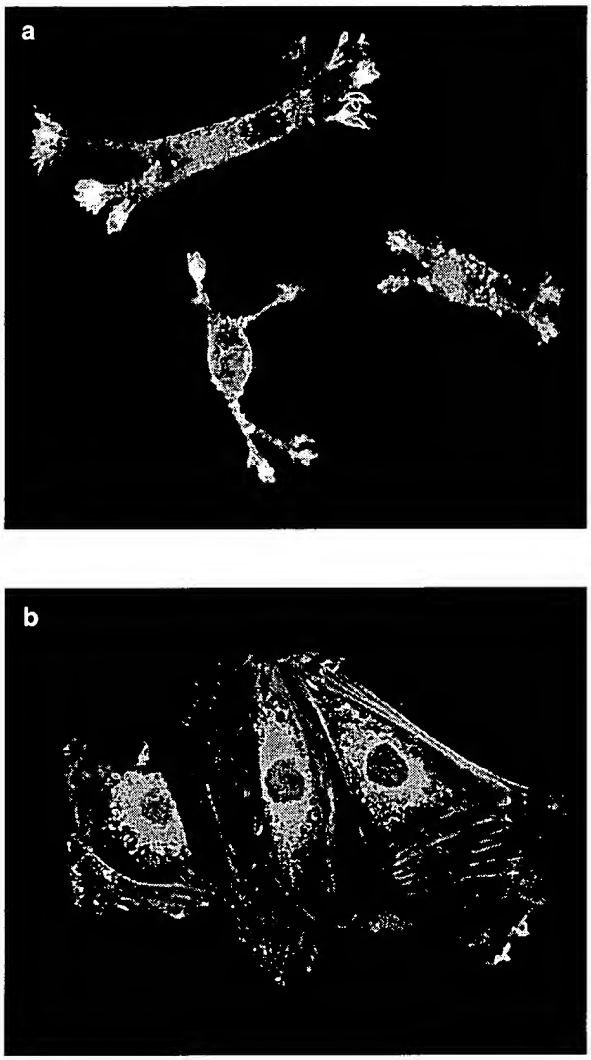

Figure 3 Changes in the arterial smooth muscle cell cytoskeletal actin distribution in A10 cells cultivated on native and denatured collagen. Double-fluorescent label studies of F and G actins were performed using phalloidin (rhodamine) as a marker of F actin, and nuclease-I (FITC) as a marker of G actin. Cells grown on native collagen (a) were relatively elongated and displayed prominent colocalization of F and G actins in focal adhesions in confocal images, while on denatured collagen (b), there was a marked qualitative increase in G-actin intensity and concentration both in the cytoplasm and around the nucleus, with prominent F-actin stress fibers at the cell perimeter. Confocal fluorescent photomicrographs, × 400.

Discussion

Our cell culture results indicate that $\alpha_v\beta_3$ integrin interactions with their cognate ligands (ie denatured collagen and TN-C) may be useful for enhancing plasmid DNA transfection. Rat arterial smooth muscle cells (A10) have been previously utilized by our group for cell culture modeling studies of gene-delivery stent functionality and mechanisms.[1,12] β_3 and $\alpha_v\beta_3$ integrins were shown by others to be upregulated in SMCs after vascular injury.[13,14] Accordingly, SMC $\alpha_v\beta_3$ integrin interactions with the ECM have been widely investigated in tissue culture and *in vivo* in order to define their role in SMC proliferation and migration mechanisms.[13,15,16] In the present study, our working hypothesis was that these same $\alpha_v\beta_3$ integrin interactions could also be involved in regulating plasmid-DNA transfection. Our integrin-blocking antibody results strongly support this (Figures 1e, 2b, 5c). Receptor signaling[9] is involved in mediating cytoskeletal changes such as those observed in the present studies. Our data also indicate that actin-cytoskeletal changes may be in part responsible for the increased transfection of SMCs cultivated on either denatured collagen or TN-C-enriched substrates compared to native collagen. We hypothesized that modification of the G-/F-actin cytoskeletal distribution might play a role in increasing transfection. G actin is an inhibitor of nuclease-I.[17] Thus, switching the cytoskeleton balance in favor of G actin or modifying the cellular actin distribution (see Figure 3b) might enhance DNA transfection based on decreased plasmid destruction.

Could the increased transfection observed in these studies be somehow related to increased proliferation observed on some of the substrates, such as denatured collagen or TN-C? The results of our cytochalasin-D experiments indicate that proliferation may not be involved in the mechanisms responsible for the observed increase in transfection (Figure 4b). In these studies, although there were no significant differences in proliferation comparing SMCs grown on native collagen to those grown on native collagen with added cytochalasin-D (see Results, above), there was significantly greater transfection (18 vs 2%) in the cytochalasin-D-treated group (Figure 4b), indicating that an actin-related mechanism may be a critical component, and that cellular proliferation-related events are occurring independently of transfection-enhancement mechanisms. This view was further confirmed through studies using the F-actin stabilizing agent, jasplakonilide, which blocked the cytochalasin-D-related increase in transfection (Figure 4b), thus providing support for an actin-related mechanism being responsible for the observed increased transfection.

A previous study by our group using a pig coronary stent angioplasty model demonstrated successful coronary artery gene transfer with a plasmid DNA delivery stent using a PLGA emulsion coating,[1] but with far less neointimal expression (1%) than observed in the present study (10.4%). These very different results arose from similar study designs: The same vector was used in both studies; comparable DNA loadings were used in both; an identical PLGA component was used in both stent formulations; both studies used Crown Stents; the animal strain, size, sex, and supplier were the same; the study durations were identical; and the operating team included all the same individuals. However, direct comparisons with our previous study[1] are not possible. Nevertheless, the increased transfection noted the present report suggests that the denatured-collagen component of the coating may have been responsible for the relatively greater transfection than that observed previously.[1] At this time, no other researchers have reported results with plasmid DNA delivery stents.

In conclusion, the present results support the view that plasmid DNA delivery from stent coatings containing $\alpha_v\beta_3$ integrin ligands, such as denatured collagen, may result in increased levels of arterial SMC transgene expression. Overall, our data indicate that this may be due to integrin-signaling mechanisms, and associated G-actin-related cytoskeletal changes.

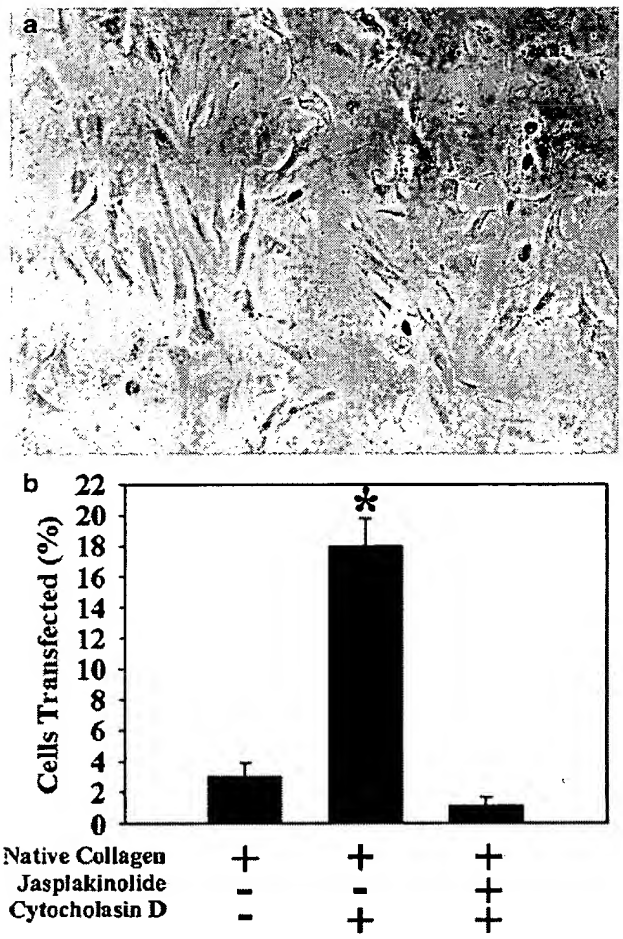

*Figure 4 Transfection and cytoskeletal manipulation. Cells grown on native collagen after treatment with the F-actin depolymerization agent, cytocholasin-D (cyto-D) (a), show increased transfection compared to a native collagen control (see in comparison Figure 1a). (a) Bright-field photomicrograph of X-gal-stained culture, ×200 magnification. (b) Quantitation of transfection, showing significant increase (*P<0.001) in transfection in the cyto-D cultures and that the blockade of the cyto-D effect with jasplakonilide results in a low transfection rate, comparable to native collagen (see also Figure 1e).*

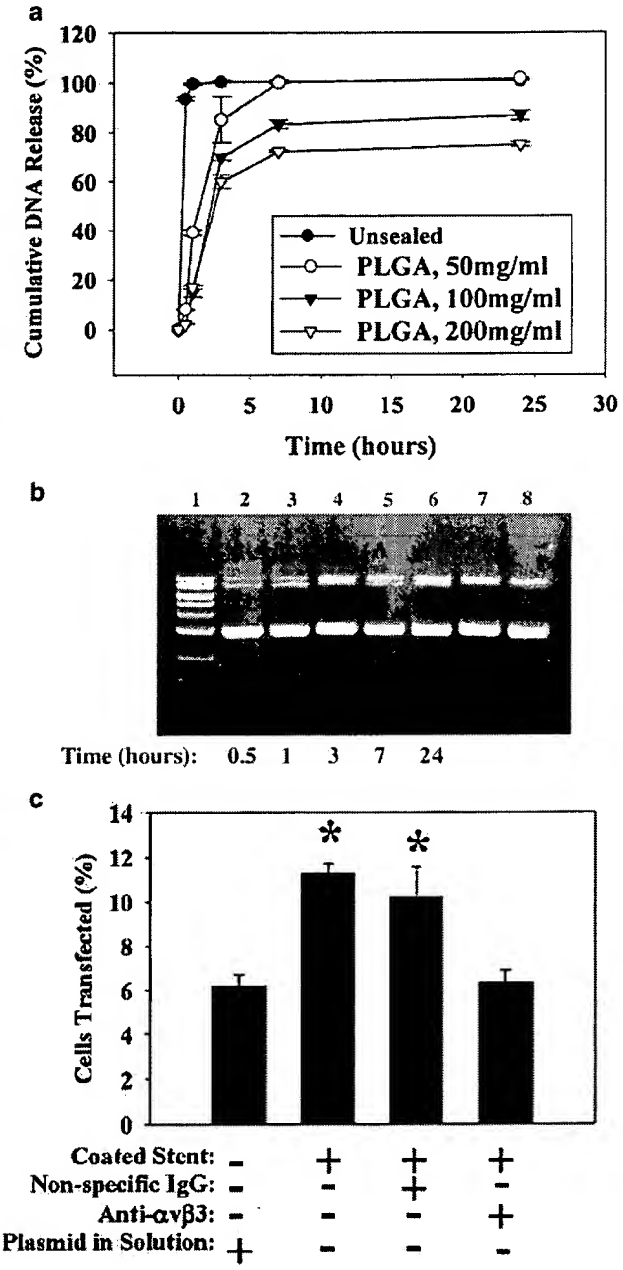

*Figure 5 Characterization of denatured-collagen-coated vascular stents. (a) Cumulative DNA release from stents coated with varying concentrations of PLGA shows controlled release of DNA load over 24 h as a function of the PLGA coating. (PLGA concentrations: closed circles=0 mg/ml; open circles=50 mg/ml; closed triangles=100 mg/ml; open triangles=200 mg/ml). (b) Agarose gel of DNA eluted from a denatured-collagen-coated vascular stent (a, 100 mg/ml PLGA condition) (lanes 1–6) shows the integrity of delivered DNA compared to control plasmid stock over time (lanes 7 and 8). Lane 1; 1 kb DNA ladder. (c) Transfection of A10 cells in vitro by contact with coated stents is dependent on stent coating and cell-$\alpha_v\beta_3$-integrin contact, as demonstrated with anti-integrin antibodies. *P<0.001.*

Materials and methods

Collagen preparation

Native collagen: Type I collagen (3.1 mg/ml, Vitronectin 100, Cohesion Technologies, Palo Alto, CA, USA) was neutralized with 0.1 N NaOH and 10×PBS to a concentration of 2.5 mg/ml, and gelled at 37°C according to the manufacturer's directions. Denatured Collagen: Type I collagen was acidified with 0.17% glacial acetic acid (v/v), boiled for 1 h, then neutralized as above. Denatured collagen was allowed to air-dry on the desired surface. In experiments testing the effect of TN-C (Gibco) on transfection, TN-C was mixed into the native collagen solution at the indicated concentrations prior to well coating, and gelled at 37°C.

Transfection in vitro

A fetal rat arterial SMC line (A10, American Type Culture Collection, Rockville, MD, USA) utilized in previous studies modeling gene-delivery stents[1,12] was used for all tissue culture experiments, and was routinely maintained in growth medium consisting of M199 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (PS, Gibco). A10

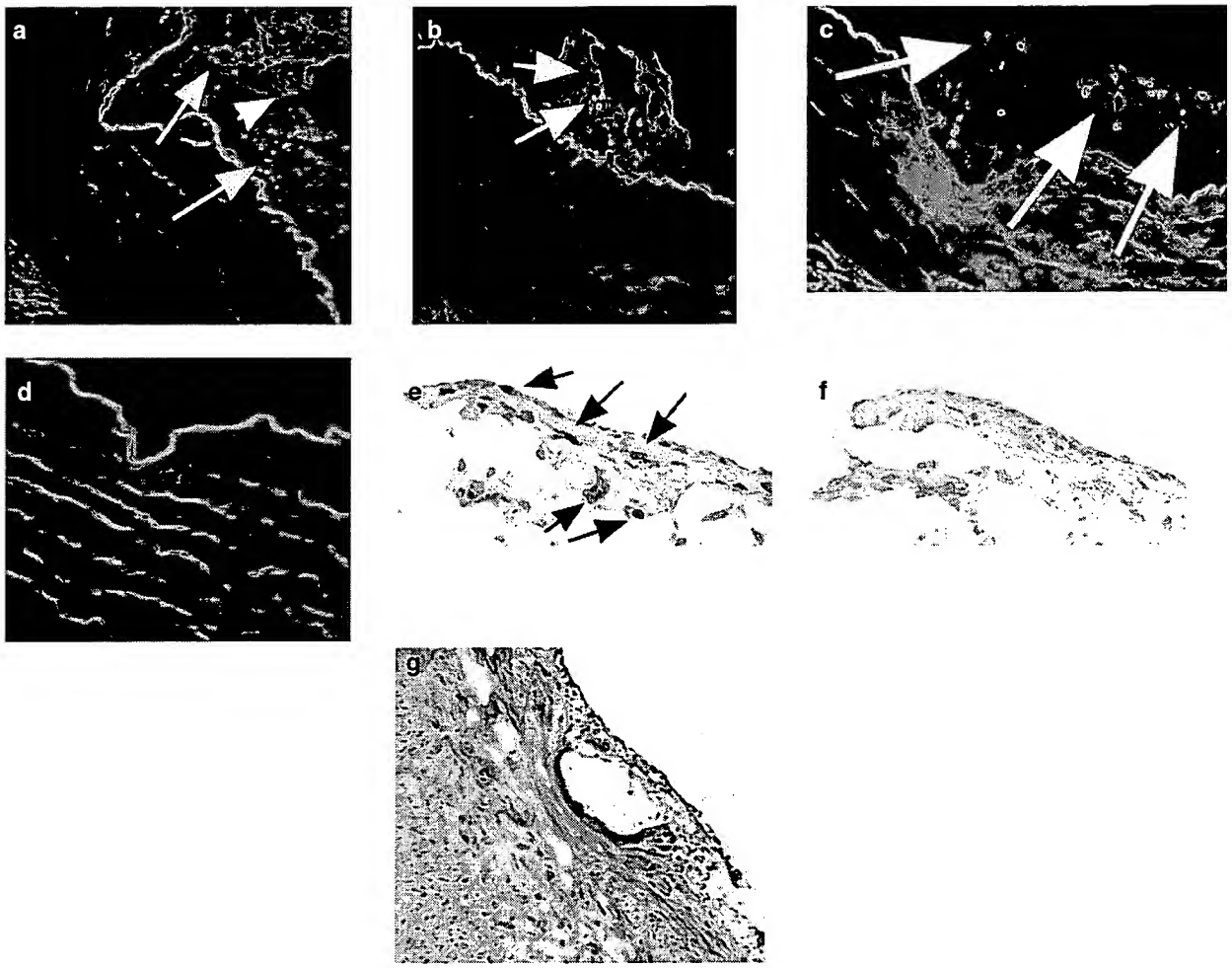

Figure 6 Plasmid delivery in vivo *using denatured-collagen-coated vascular stents. (a–c) FITC-fluorescent micrographs showing examples of GFP reporter gene expression 7 days after stent deployment, with enhanced neointimal GFP (arrows) expression. GFP expression is absent in arteries stented without GFP plasmid (d). Serial sections of neointima shown in (c) are the subject of confirmatory GFP (arrows) immunohistochemistry shown in (e), in which purple (vector VIP, see Materials and Methods) denotes GFP-positive cells, and negative control immunohistochemistry (nonimmune IgG), shown in (f). (g) Hematoxylin and eosin of stented artery showing early neointimal formation prominent around the site of a stent post and inflammation of the stented arterial media (original magnifications* × *200).*

cells were plated at 1×10^5 cells/well in six-well plates 20 h in advance of transfection. Transfection of A10 cells was performed using plasmid DNA and Lipofectamine (Gibco) according to the manufacturer's directions. Typical formulations used 2.5 μg plasmid DNA (β-galactosidase, pNGVL1-nt-β-gal, University of Michigan, Ann Arbor, MI, USA), with 5 μl Lipofectamine. Cells were fixed at 72 h using 4% paraformaldehyde.

Integrin blocking

A10 cells were pretreated with either mouse monoclonal anti-rat β_3 integrin IgG (25 μg/ml, CD61*, PharMingin, San Diego, CA, USA), mouse monoclonal anti-human $\alpha_v\beta_3$ integrin IgG (15 μg/ml, LM609, Chemicon, Temecula, CA, USA), or with equivalent concentration of nonimmune mouse IgG* (Pierce, Rockford, IL, USA) at the time of plating as previously described[9] (*sodium azide was removed by microdialysis (Slide-A-Lyzer, MWCO 10 kDa, Pierce, Rockford, IL, USA)). Cells were suspended at 1×10^5 cells/ml in growth medium containing the antibody, and then plated. Incubations and transfection then proceeded in the same manner as for nontreated cells.

β-Galactosidase expression

Medium was aspirated from cultures, which were then fixed with 4% paraformaldehyde. This in turn was rinsed twice from the cultures with 2 mM $MgCl_2$ in PBS, and replaced with X-gal stain.[18] Cultures were incubated overnight at 37°C, and then washed three times with PBS. The total number of cells and the number staining positive for X-gal were visually counted per × 100 field, and the per cent positive per well determined from the mean of 10 fields per well.

Cell attachment, proliferation, and viability

Triplicate cultures plated under conditions as described were incubated in six-well plates for 5 h at 37°C prior to visual counting of cells attached in at least three × 100 fields (10.7 mm^2) per well for quantification of attachment. Cell number per mm^2 was again determined in this manner at 72 h after treatments as described. Cell

growth was also quantified in parallel 96-well plates using a commercially available kit based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases in viable cells (WST-1, Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's directions. Trypan blue exclusion and cell count per field were performed in parallel cultures to determine culture viability, and confirmed using a Live/Dead Assay™ (Molecular Probes, Eugene, OR, USA), which is based on the ability of viable cells to enzymatically convert calcein AM to fluorescent calcein, as per the manufacturer's directions.

Double-fluorescent labeling

Cells were fixed with 4% paraformaldeyde at 6 h. Cultures were then stained for either F or G actin (Rhodamine 514 phalloidin, or Oregon green 488 Deoxyribonuclease I, respectively, Molecular Probes), following permeabilization and blocking, according to the manufacturer's directions. Cultures were then mounted using Vectashield mounting medium containing 4,6-diamidino-2-phenylindole mount (DAPI, Vector Labs, Burlingame, CA, USA) for visualization of nuclei, and confocal images recorded using a Leica TCS4D confocal microscope at $\times 400$ magnification.

Cytoskeletal manipulation

Cells were allowed to adhere to native collagen-coated six-well plates as above, then growth medium was replaced by serum-free M199, and transfection with plasmid allowed to proceed for 3–5 h. The medium was changed again to M199/2% FBS containing either 10 μM Jasplakinolide (jas, Molecular Probes) or 0.7% methanol (vehicle). Cells were incubated for 30 min and medium again replaced with M199, without or with 100 nM cytocholasin-D (cytoD, Sigma, St Louis, MO, USA) for an additional 30 min. After another medium change to remove jas or cytoD, incubations proceeded in the same manner as for nontreated cells, in M199/2% FBS, and transfection evaluated at 72 h. In order to evaluate the effect of cytoskeletal manipulation with jas and cytoD on the growth of the cells on collagen, cells were plated as above on native collagen and treated with cytoD or jas as above. After the treatments, the medium was replaced with growth medium for 72 h and assayed with WST-1 as above.

Stent-coating formulation

Denatured collagen was mixed with 10% (w/w) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, St Louis, MO, USA) as a crosslinking agent to achieve a robust coating. This mixture was incubated at 37°C for 30 min prior to use as a stent coating. GFP Plasmid DNA (pEGFP-N3, Clontech, Palo Alto, CA, USA) was added to the above collagen solution and stored at 4°C. The formulation was such that fully coated stents carried 500 μg DNA. The mixture was applied in 10 μl increments under sterile conditions onto the stent surface of 15 mm Crown stents (Cordis, Warren, NJ, USA), with manual rotation, to form a thin, uniform coating, and dried at 37°C under vacuum. The procedure was repeated until a total of 400 μl (1 mg of collagen) was coated per stent. Coated stents were dipped briefly in 100 mg/ml PLGA (MW 50 000, Birmingham Polymers, Birmingham, AL, USA) and air-dried. This thin coating was shown to be optimal for preventing rapid DNA release by preliminary experiments in which *in vitro* DNA release from stents coated with designated concentrations of PLGA was characterized.

Collagen-plasmid DNA-coated stents were shaken in 100 μl TE buffer (pH 7.4) at 37°C. Buffer was removed and replaced with fresh buffer after 0.5, 1, 2, 3, 7, and 24 h of shaking. The DNA concentrations in the collected buffers were determined by UV spectrometry (GeneQuantPro, Amersham Pharmacia Biotech), and checked for structural integrity by running 0.3 μg/lane on an agarose gel in comparison to untreated plasmid.

Transfection via stent in vitro

A10 SMCs were prepared in uncoated six-well plates as described above, prior to transfection. Coated stents were incubated in 300 μl serum-free medium at 37°C for 20–30 min with the addition of 5 μl Lipofectin (GIBCO) for 15 min. After aspiration of medium from the cell cultures, stents and their incubation medium, diluted to 1 ml, were transferred to the cell cultures and incubated at 37°C for 5 h. FBS was then added to a final concentration of 5%, and changed to growth medium after 24 h. After fixation, cultures were DAPI mounted for visualization of nuclei, and total cell number determined per $\times 200$ field in immediate contact with the stent. GFP-expressing cells were also visually counted in the same fields, and results reported as the percentage of cells transfected (mean $\pm$ s.e.) of at least five fields per culture in replicate cultures.

Transfection via stent: in vivo

Animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. Denatured-collagen–PLGA-coated stents (either with GFP-plasmid loading or control without vector), prepared as described above, were deployed using two stents per animal in both the left ascending or left circumflex coronary arteries (LAD, LCX) of seven normal Yorkshire swine as previously described by our group.[1] Animals were euthanized after 7 days and representative samples of stented arteries, arterial segments distal to the stents, and unstented control pig coronary arteries were prepared for morphology studies by first removing the stent from the arteries and then embedding in frozen section media (O.C.T., Tissue-Tek, Torrance, CA, USA). The percentage of GFP-positive cells, normalizing for DAPI-positive cells, in representative frozen sections of each of the 14 stented arteries and nonstented arteries was determined according to previously published methodology, separately reporting neointimal and medial expression.[1,12] GFP expression was confirmed with anti-GFP immunohistochemistry,[1,12] and hematoxylin and eosin sections were prepared for routine light microscopy.

Statistical analysis

Data for all experiments were expressed as means plus or minus the standard error of the mean (s.e.). The significance of differences was assessed using Student's *t*-test, and was termed significant when $P \leq 0.05$.

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